Decreased Diversity of the Fecal Microbiome in Recurrent *Clostridium difficile*–Associated Diarrhea

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Antibiotic-associated diarrhea due to *Clostridium difficile* (CDAD) is thought to reflect colonization of a disrupted microbial community by the pathogen. We profiled the fecal microbiota of patients with CDAD (both initial and recurrent episodes) by culture-independent phylogenetic analysis of 16S rRNA-encoding gene sequences. Compared with those from control subjects and patients with an initial episode, the fecal communities in patients with recurrent CDAD were highly variable in bacterial composition and were characterized by markedly decreased diversity. Preservation and restoration of the microbial diversity could represent novel strategies for prevention and treatment of recurrent CDAD, which is often recalcitrant to existing therapies.

Diarrhea is a common side effect of the administration of antibiotics, with *Clostridium difficile* being responsible for most of the severe cases of antibiotic-associated diarrhea (AAD), including the development of pseudomembranous colitis [1]. Although *C. difficile* colonizes a minority of healthy individuals, it has been suggested that, in patients who develop AAD due to *C. difficile* (CDAD), administration of antibiotics either allows colonization by *C. difficile* after ingestion of environmental spores or permits overgrowth of indigenous *C. difficile* [2].

Standard treatment for CDAD involves the administration of antibiotics that suppress *C. difficile* including metronidazole or vancomycin. Although the majority of cases will respond initially to either drug, recurrence after the discontinuation of anti-*C. difficile* treatment can occur [3]. Treatment of recurrent CDAD (RCD) can be difficult and has led to dramatic interventions, such as the administration of donor stool from healthy volunteers [4].

The mechanisms by which antibiotic administration leads to the development of CDAD are not entirely clear. The human gut is colonized by a diverse community of microorganisms that exist in a complex symbiosis with their host [5]. Until recently, it has been difficult to fully appreciate the diversity of this microbial community, but the application of molecular techniques has started to reveal the structure and function of the gut microbiota, independent of the need for culture [5].

We recently have reported on the use of molecular methods involving retrieval of 16S rRNA-encoding gene sequences to follow the changes in the gut microbiota that can lead to AAD that is not associated with *C. difficile* [6]. In the present study, we applied 16S rRNA-encoding gene sequence analysis to profile the community structure of the gut microbiota of patients with either initial episodes of CDAD (ICD) or RCD.

Subjects, Materials, and Methods. All fecal specimens submitted to the microbiology laboratory for detection of *C. difficile* toxin were considered for inclusion in the present study. If the sample assumed the shape of the container, and thus was considered to represent diarrhea, 200-mg samples were collected and immediately were frozen at −70°C. The specific toxin A/toxin B ELISA (Tech LAB ELISA; Wampole) was used to screen stool samples for the presence of *C. difficile*. Healthy control subjects were recruited from patients who visited the Michigan State University Outpatient Clinic for routine care. All control subjects were ≥65 years of age and free of malignancy or gastrointestinal disease. They had not taken antibiotics for ≥3 months before stool collection. Stool samples from control subjects were also tested for the presence of *C. difficile* toxin. The protocols for the present study were approved by the Institutional Review Boards of Michigan State University and the E.W. Sparrow Hospital.

Analysis of the 16S rRNA-encoding gene clone library was performed as described elsewhere [6]. In brief, DNA extracted

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from frozen fecal samples was amplified by polymerase chain reaction (PCR) primers (8F, 5’-AGAGTTTGATCCTGGCAG-3’; 1492R, 5’-GGTTACCTTGTTACGACTT-3’) that target conserved regions of the 16S rRNA-encoding gene [7]. Purified amplicons were ligated into a plasmid vector and were used to transform *Escherichia coli*. Cloned inserts from randomly selected colonies were amplified directly from overnight cultures, by use of vector-specific primers. Partial 16S sequences were determined by a single sequencing run using the 8F primer. Raw sequence data were processed through an automated “information pipeline” available through the Ribosomal Database Project (RDP) II Web site (http://rdp.cme.msu.edu/). Automated quality scoring, trimming, and chimera screening were performed. The sequences have been deposited in GenBank, under accession numbers E197217–E199078. Screened sequences were aligned, and a distance matrix based on this alignment was generated.

The distance matrix was fed into DOTUR [8] to group the sequences into operational taxonomic units (OTUs), calculate ecological diversity measures (including the Shannon index [9]), and generate rarefaction curves by use of an OTU definition of 97% sequence similarity. Taxonomic assignment was performed by SeqMatch and Classifier via the RDP II Web site. Statistical comparisons of the Shannon diversity index were performed by use of analysis of variance with an α value set to 0.05.

**Results.** A total of 1427 partial 16S rRNA-encoding gene sequences were obtained from 10 individuals—7 patients with CDAD and 3 control subjects. The CDAD patients included 4 with ICD and 3 with RCD (table 1, figure 1). To compare the microbial communities in each individual, each microbiome was characterized at the level of bacterial division (phylum). In the 3 control subjects and the 4 patients with ICD, the majority of the 16S rRNA-encoding gene sequences represented organisms within the phyla Bacteroidetes and Firmicutes. For the patients with RCD, the composition of the microbiota was more variable and deviated from the normal predominance of Bacteroidetes and Firmicutes (figure 2A). 16S sequences corresponding to *C. difficile* were encountered only in the microbiome of individuals who had *C. difficile* toxin detected by ELISA, including 2 of the 4 patients with ICD and 2 of the 3 patients with RCD (table 1). In the 2 patients with ICD, the organism represented 1.6% or 3.1% of the total sequences identified, whereas, in the 2 patients with RCD who had detectable *C. difficile* 16S sequences, the percentages were 8.9% and 31% of the total. All patients who had *C. difficile* toxin detected by ELISA had *C. difficile* 16S sequences detectable by an end-point PCR assay (data not shown).

The Shannon-Weiner diversity index for the 3 control subjects was similar to that for the 4 patients with ICD (*P* = 0.252) but was significantly higher than that for the 3 patients with RCD (*P* = 0.0154). Patient ICD4 was an exception: although suffering from ICD, this patient’s Shannon-Weiner diversity index was lower than both that for the 3 control subjects and that the 3 remaining patients with ICD.

Overall species richness (i.e., the total number of phylotypes) of each microbiome was compared by the construction of rarefaction curves. Richness in the patients with ICD was similar to that in the control subjects, with the shape of the curve revealing

| Table 1. Patient demographics and summary of 16S clone libraries. |
| --- | --- | --- | --- | --- |
| **Initial episode CDAD** | **Age** | **Sex** | **Antibiotic(s) a** | **Total sequences, no.** | **OTUs, no.b** | **Clostridium ** | **Shannon** |
| | | | | | | **difficile** | **index** |
| ICD1 | 82 | Male | MET, LEV | 130 | 29 | 3.1 | 2.84 |
| ICD2 | 87 | Male | P/T, CTX, AZM | 131 | 28 | 0 | 2.77 |
| ICD3 | 84 | Female | VAN, CTX, AZM | 136 | 36 | 0 | 3.04 |
| ICD4 | 79 | Female | IMP, LEV, P/T | 184 | 23 | 1.6 | 1.68 |
| **Recurrent CDAD** | | | | | | | |
| RCD1 | 83 | Male | NFN, T/S, VAN | 137 | 12 | 31 | 1.97 |
| RCD2 | 84 | Male | MET | 168 | 11 | 8.9 | 1.56 |
| RCD3 | 80 | Male | MET | 142 | 11 | 0 | 1.15 |
| **Control subjects** | | | | | | | |
| C1 | 67 | Male | | 134 | 51 | 0 | 3.57 |
| C2 | 71 | Female | | 125 | 27 | 0 | 2.54 |
| C3 | 65 | Female | | 140 | 45 | 0 | 3.38 |

**NOTE.**  AZM, azithromycin; CDAD, antibiotic-associated diarrhea due to *C. difficile*; CTX, ceftriaxone; IMP, imipenam/cilastatin; LEV, levofloxacin; MET, metronidazole; NFN, nitrofurantoin; OTU, operational taxonomic unit; P/T, piperacillin/tazobactam; T/S, trimethoprim/sulfamethoxazole; VAN, vancomycin.

a Administered immediately before collection of *C. difficile*-positive stool.

b Based on 97% sequence similarity.

The figure is available in its entirety in the online edition of the *Journal of Infectious Diseases*.

**Figure 1.** Time line of administration of antibiotics to patients.
that the total richness of the microbial community had not been completely sampled. However, the species richness in the patients with RCD was consistently lower than both that in the patients with ICD and that in the control subjects (figure 2B).

Once again, patient ICD4 was notable: species richness, as judged by rarefaction analysis, was lower in this patient than in either the control subjects or the other patients with ICD.

Because of the discordant results for patient ICD4, additional clinical information was obtained for this patient, who initially was treated with a 10-day course of metronidazole administered by mouth. A follow-up ELISA for detection of *C. difficile* toxin, performed after treatment, was negative. However, 10 days after discontinuation of metronidazole, the patient developed recurrent diarrhea. The patient was not being treated with any antimicrobial drugs at that time, but a repeat ELISA for detection of *C. difficile* toxin was positive. Examination of the records of the remaining 3 patients ICD revealed that none of them had recurrence of CDAD up to 12 months after initial treatment for CDAD.

**Discussion.** Recently, it has been appreciated that the complex community of microbes that inhabits the mammalian gut represents an assemblage of organisms that exists in a balanced symbiosis with its host [5]. Therefore, alterations in the structure of this microbial community can have implications for the homeostasis of the host. Changes in the gut microbiome have been associated with obesity, inflammatory bowel disease and AAD. These pathologic conditions are thought to arise via changes in either the metabolic activity of the altered microbial community or the interaction between the microbiome and the host immune system.

One function that has been ascribed to the indigenous gut microbiota is that of “colonization resistance” [10]. The gut microbiota appear to be largely refractory to colonization by additional “invasive” species. The development of CDAD is thought to represent the breakdown of colonization resistance against *C. difficile* [2]. Administration of antibiotics suppresses members of the indigenous microbiota, allowing either expansion of pre-existing *C. difficile* (normally maintained at only low levels) or germination and subsequent expansion of *C. difficile* spores acquired from the hospital environment. The standard treatment of CDAD, with oral metronidazole or vancomycin, directly suppresses *C. difficile*, presumably allowing recovery of the indigenous microbiota and thereby restoring colonization resistance. An underlying assumption is that the remaining microbiome is sufficiently diverse to recover to a “normal” state. However, to date, minimal work has been performed to monitor the specific shifts in the microbial community that occur in the setting of CDAD.

RCD can occur via either recrudescence of the original strain or acquisition of a new strain after discontinuation of treatment [3]. It has also been suggested that the remaining microbiome is deficient in the ability to restore colonization resistance against *C. difficile* [3]. Therefore, if the microbiome could be intentionally manipulated to increase colonization resistance, that might represent a novel treatment for recurrent disease. There is evidence that, in some cases, RCD occurs in patients who have deficient immune responses to *C. difficile* toxin [11]. Although immunity against *C. difficile* toxin plays a role in some cases of RCD, it is also clear that colonization resistance against *C. difficile* can play a role. A number of relatively efficacious treatment regimens for recurrent *C. difficile* that invoke alteration of the microbiome—regimens including tapered, cycled, or pulsed administration of anti-*C. difficile* antibiotics or the administration of probiotic organisms—have been tried for treatment of RCD [12, 13] [14]. The likely primary effect of these interventions, or what could be considered the ultimate in microbial ecologic treatment—that is, transferring fecal bacteria from healthy
individuals to patients with RCD [4, 15]—is to alter the patient’s microbiome and to restore colonization resistance.

The data presented here provide evidence that RCD is associated with decreased overall diversity of the gut microbiota. By analyzing hundreds of clones, we compared the composition of the more abundant members of the microbial community, rather than completing an exhaustive survey of the gut microbiome. We observed that the fecal microbiome of patients with RCD was consistently and significantly decreased in phylotype richness, compared with that of the control subjects and the patients with ICD. This markedly decreased richness was not encountered in patients with AAD that was not associated with C. difficile (figure 3).

Furthermore, the distribution of phylotypes within the microbiome of the patients with RDC was altered (figures 2A and 4). Data from a number of studies indicate that, in healthy normal individuals, the vast majority of bacteria belong to either the Bacteroidetes or Firmicutes phylum. The dominance of these 2 bacterial phyla is thought to represent the evolutionary development of complementary yet distinct metabolic roles in the gut ecosystem [5]. Thus, the decreased overall richness and altered distribution of 16S phylotypes seen in the patients with RCD indicate a significant deviation from the normal state. Evidence that this deviation may result in a loss of colonization resistance is provided by the observation that, in 2 of the 3 patients with RCD, 16S phylotypes corresponding to C. difficile itself became dominant members of the microbial community. This finding supports the results of previous animal and in vitro studies, which have demonstrated that the size of the C. difficile population is controlled by the gut microbiota [2].

It was fortuitous that 1 individual studied during ICD later developed RCD. Interestingly, although this patient still had a predominance of Bacteroidetes and Firmicutes, the overall 16S phylotypes’ richness was at a level intermediate between the richness measured both in the control subjects and in patients with ICD and the reduced richness seen in the patients with RCD. Reduced phylotype richness during ICD may be a marker of patients who may be at risk for development of RCD. Formally testing this hypothesis will require additional prospective studies.

In summary, we have presented molecular-based ecologic evidence for the role that decreased microbial diversity plays in cases of RCD. This finding could lead the way to new treatments and preventive measures against this reemerging infectious disease.

References

Figure 1. Time line of administration of antibiotics to patients. Results shown are for patients with an initial episode of antibiotic-associated diarrhea due to *C. difficile* (ICD) and patients with recurrent antibiotic-associated diarrhea due to *C. difficile* (RCD). The duration and timing of administration of antibiotics before day 0 (i.e., the day of collection of stool for inclusion in the study) is depicted. For patients with RCD, the day of initial diagnosis of *C. difficile* toxin by ELISA is shown. The gold asterisks (*) denote previous stool samples that had been positive for *C. difficile* (only patients with RCD). AZM, azithromycin; CTX, ceftriaxone; CTZ, ceftazidime; IMP, imipenem/cilistatin; LEV, levofloxacin; MET, metronidazole; NFN, nitrofurantoin; P/T, piperacillin/tazobactam; TMP-SMX, trimethoprimsulfamethoxazole; VAN, vancomycin.
Figure 3. Rarefaction analysis comparing overall diversity of indigenous microbiota in healthy control subjects and patients. Results are shown for control subjects (Control), patients with an initial episode of antibiotic-associated diarrhea due to Clostridium difficile (ICD), and patients with recurrent antibiotic-associated diarrhea due to C. difficile (RCD), as well as in 4 patients with antibiotic-associated diarrhea not due to C. difficile (nonCDAD). The 16S sequences of all patients in each group were pooled, with an operational taxonomic-unit definition set at 97% sequence identity. Each rarefaction curve is plotted, along with its 95% confidence interval (95%ci). As was seen in the primary analysis, the overall diversity in patients with RCD was significantly lower than that in control subjects, patients with ICD, and patients with nonCDAD.

Figure 4. Dendrograms comparing the operational taxonomic unit (OTU) composition of the 10 subjects in the study, based on calculation of the Bray-Curtis shared-species distance metric [9]. At an OTU definition of 97% sequence similarity, there is minimal clustering of the microbial communities in each patient with a high average Bray-Curtis distance, reflecting a high level of species- and strain-level variation, as has been observed by Ley et al. [5]. However, at a higher level of taxonomic classification, with an OTU definition of 80%, there is clustering of microbial communities from the control subjects (C) and the patients with an initial episode of antibiotic-associated diarrhea due to Clostridium difficile (ICD). The microbial communities in patients with recurrent antibiotic-associated diarrhea due to C. difficile (RCD) are more varied and more distinct from each other and the microbial communities in the other 2 classes of individuals. This provides additional analysis of differences in the composition of microbial communities depicted in figure 2A.